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Confirmation of cross-pollination of *Ardisia crenata* by sequence-characterized amplified region (SCAR) markers

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Abstract

To investigate the origin of seven *Ardisia crenata* Sims seedlings with non-variegated foliages (VSm) from a progeny of a mother plant with variegated foliage and red berries (VM), morphological and genetic characteristics of these seedlings were compared with mother plants of *A. crenata* with VM, plants with non-variegated leaves and white berries (WM), and plants with non-variegated leaves and red berries (RM). Genetic data include randomly amplified polymorphic DNA (RAPD) markers and sequence analysis of an unidentified locus that was obtained from seven VSm seedlings out of 261seedlings of VM with variegated foliage (VS) seedlings, WM, and seedlings from WM (WS). RAPD analysis indicates that VM, WM, and progeny populations VSm and WS are more closely related to each other than to RM and RM progeny (RS). Substitution in a 374-base long nucleotide sequence revealed that WM and most of WS and VSm produced similar sequence data with some exceptions, such as seedling VSm 2 and 5 showing polymorphisms at positions 7 (C replacing T) and 243 (A replacing T). Based on the RAPD and the sequence analysis for the VSm and WM specific band, it is concluded that these seven VSm seedlings were resulted from cross-pollination between VM and WM. Hybrid origin of VSm seedlings between VM as a maternal source and WM as a paternal source is verified by polymerase chain reaction (PCR) utilizing sequence-characterized amplified region (SCAR) markers (forward primer, ARD-1-F; GGACTGGAGTAGAGGATAGAGTTTTG and two reverse primers, ARD-2-R; GGACTGGAGTGCTCTATGAATTG and ARD-3-R; TGTCAGCAGCCTACCACTAGC). These SCAR markers were successful to identify VM progenies with non-variegated leaves involving WM as a paternal source.
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Keywords: Randomly amplified polymorphic DNA (RAPD); Sequence-characterized amplified region (SCAR) markers; Single nucleotide substitution; Nucleotide sequencing

1. Introduction

The genus *Ardisia* consists of more than 200 species growing in the warm climates of tropical and subtropical regions of both hemispheres (Bailey, 1925). *Ardisia* species have been used as outdoor ornamental or indoor houseplants because of their bright red berries. *Ardisia crenata* Sims is small evergreen shrub most commonly with non-variegated foliage and red berries (Lee, 1998). However, plants with variegated foliage and red berries or with regular leaves and white berries are rare, but available in the trade (Lee et al., 2002).

Seed reproduction biology of *A. crenata* and whether or not they are self-pollinated has not been investigated in detail. However, the possibility of cross-pollination should not be excluded in the greenhouse since in nature, a low out-crossing rate in a small population and a high out-crossing rate in a large population of *A. crenata* var. *bicolor* was reported (Chen et al., 2001). The rate of cross pollination of *A. crispa* (*A. crenata*) 'Takarabune' was also reported to be high (Sekine, 1994). In greenhouse environments over the past 10 years, seedlings collected from a group of plants phenotypically identical in regards to berry color and foliage shape were true to the type of their mother plants suggesting out-crossing in the greenhouse did not occur.

When seeds from one *A. crenata* plant with variegated and undulated leaves (VM) were germinated in a greenhouse, seven

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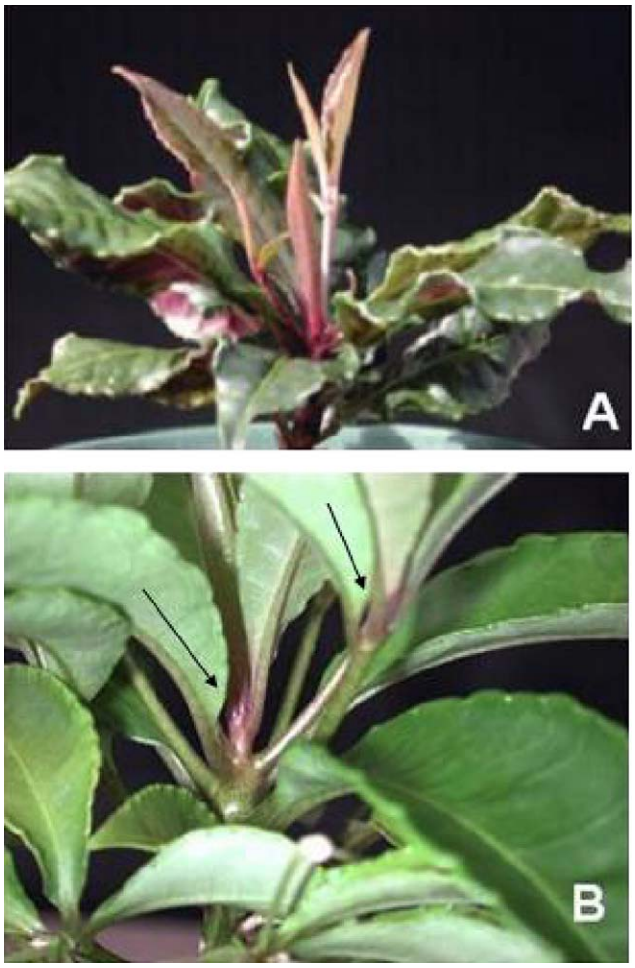

Fig. 1. *Ardisia crenata* seedlings with variegated foliage (VS) and morphologically undistinguishable from the mother stock plant (VM) (A) and seedlings with non-variegated foliage (VSm 3) and morphologically distinguishable from VM (B). Shoot apices of VSm 3 seedling showing a red color is indicated with an arrow.

out of 261 seedlings produced smooth, undulated, and non-variegated leaves (VSm) (Fig. 1). Leaves were very similar to those of RM and its seedlings (RS) and also WM and its seedlings (WS). During flowering of VM, many *A. crenata* plants with red berries (RM) and one plant with white berries (WM) were flowering in the same air-conditioned greenhouse. The seven VSm seedlings could, therefore, result from cross-pollination with WM or RM, but the possibility of segregation from VM could not be excluded. These questions could not be answered by observing morphological characters, but may be answered using molecular markers generated by DNA-based analysis such as randomly amplified polymorphic DNA (RAPD) (Williams et al., 1990), amplified fragment length polymorphism (AFLP) (Joung and Roh, 2004; Vos et al., 1995), or single nucleotide polymorphisms (SNP) (Brookes, 1999). It takes about 4 years for seedlings to flower and produce berries. Therefore, the hybrid origin or segregation of VSm could only be answered at an early growth stage by molecular markers. Developing the sequence-characterized amplified region (SCAR) markers based on RAPD or other molecular markers would overcome the reproducibility problems associated with the RAPD techniques (Paran and Michelmore, 1993). The SCAR markers obtained by cloning the significant RAPD bands were used to identify olive (*Olea europaea* L.) tree cultivars (Bautista et al., 2002) and SCAR marker mapping of AFLP markers linked to seed coat color loci in *Brassica juncea* (L.) Czern has been developed to produce yellow-seeded lines (Sabharwal et al., 2004) by means of marker-assisted selection.

The objective of this study was (a) to use RAPD markers to investigate whether these seven VSm seedlings resulted from a cross pollination with RM or WM, (b) to identify a VSm-specific marker band and to examine any changes in the nucleotides present in the marker band, and (c) to construct SCAR markers to confirm the hybrid origin of VSm.

2. Materials and methods

2.1. Plant materials

One *A. crenata* plant with variegated leaves and red berries (VM), 25 plants of non-variegated leaves and red berries (RM), and one plant of non-variegated leaves with white berries (WM) were grown in an air conditioned greenhouse maintained at 18.5/18.0 °C. Seeds from these mother stock plants were collected and sown on 11 February 2002 and germinated in the same greenhouse. Seedlings were transplanted to10 cm pots filled with ProMix BX (Stamford, CN, USA) on 16 February 2003. Slow release fertilizer (14N–6P–11.4K) was applied at a rate of 0.8 g per pot on 11 August 2003. During the culture, seedlings received 200 ppm N from a 15N–7P–14K water soluble fertilizer once a month.

2.2. Genomic DNA extraction and PCR amplification

Total genomic DNA was extracted from young leaves from a total of 58 samples comprised of one of each RM, WM, and VM, and seedlings of 7 VSm, and 16 of each from RM, VM, and WM using DNeasy Mini Kit (QIAGEN, Inc., Valencia, CA, USA). Based on the previous work (Lee et al., 2002), 11 10-mer random primers (Operon Technologies, Alameda, CA, USA) (A-04, A-05, A-08, A-14, A-18, B-04, C-09, C-11, C-13, C-15, and C-18) were selected for analysis. Amplification was performed using 10 ng of template DNA, 5 pM of primers and ready-to-go PCR Beads (PCR bead 27-9555-01, Amersham Pharmacia Biotech, Pharmacia Biotech Inc., USA) for a total volume of 25 μl, using a PTC-100 Programmable thermal cycler (MJ Research, Watertown, MA, USA) (Joung and Roh, 2004). The amplified products were separated on a 1.7% agarose gel in TBE buffer at 105 V for 3 h and stained with ethidium bromide (5 μg/ml). In each gel, samples from RM, VM, and WM, the seven VSm seedlings, and five to six samples each of three other *A. crenata* seedlings were loaded.

2.3. Data analysis of RAPD markers

Gels were documented digitally using an Image Analyzer (AlphaImager 2000, Alpha Innotech Corporation, San Leandro, CA, USA). Bands of all gels were electronically scored using

the ONE-Dscan gel analysis software (Scanalytics, Inc., Firfax, VA). The molecular size of each fragment was estimated based on 100 bp DNA Ruler Plus (Fermentas, MD) according to the algorithm provided in the ONE-Dscan software. The amplified bands were scored as diallelic for each assigned locus (1, band present; 0, band absent) to compile a data matrix. To align co-migrating bands, a matrix was generated from the raw data for each gel based on percent migration (%rf) of the scored bands. Bands with similar rf values after correction manually were marked as homologous. Polymorphic DNA bands from the RAPD analyses were scored using the ONE-Dscan gel analysis software. P-distance was calculated based on the proportion of differences expressed as the number of different bands divided by the total number of polymorphic bands, and dendrograms were constructed using the neighbor-joining method (Saitou and Mei, 1987). Bootstrap values from 500 replications were obtained using the molecular evolutionary genetic analysis (MEGA), Version 1.0 program (Kumar et al., 1994).

2.4. Cloning and sequencing

RAPD amplifications were performed on samples of WM, VSm, and WS using RAPD primer OPB-04 which produced a band at about 390 bp. Samples of RM, VM, RS, and VS did not produce the band. Band was excised from the gel and extracted using Qiagen Gel Extraction kit (No. 28706, Qiagen, Valencia, CA). Cloning was performed using the TA Cloning kit, Version V (K2040-40, Invitrogen Technologies, Carlsbad, CA). DNA that was PCR amplified from miniprep cultures of each colony line were run on a 1.5% agarose gel and gels were stained with 0.5 μg/ml ethidium bromide. Colony PCR was performed on white colonies to verify the insertion of the PCR product. Bands at approximately 690 bp PCR product from colony PCR from each clonal line were selected and plasmid DNA extracted using the Qiagen Qiaprep Spin Miniprep kit (no. 27106, Qiagen, Valencia, CA). Following overnight cultures and growing in 2× YT broth liquid media (Qbiogene, Carlsbad, CA) at 37 °C, plasmid DNA was isolated for sequencing and a minimum of three and a maximum of 13 colonies for each individual were sequenced to construct a single nucleotide substitution table. The isolated 1 μl plasmid DNA was sequenced with 0.5 μl of BigDye Terminators Version 3.1 (Applied Biosystems, Foster City, CA), 0.3 μM of the original PCR primers, 1× of Taq DNA polymerase Buffer (Promega, Madison, WI), and 1.75 mM of $MgCl_2$ in 5 μl reaction volume. The sequencing reactions were analyzed on a 3730 Genetic Analyzer (ABI PRIZM, Applied Biosystems, Foster City, CA). Consensus sequence for each plasmid was made by aligning the sequences for the plasmid DNA. The sequence data were analyzed and aligned with the standard Phred and Phrap DNA analysis software and nucleotide polymorphisms was carried out with PolyBayes (Marth et al., 1999).

2.5. Construction of primers

Based on the sequence analysis of WM, two forward (ARD-1-F; GGACTGGAGTAGAGGATAGAGTTTTG and ARD-2-F; GGTTGGAGTTAGAGTTTTTCAGC) and three reverse (ARD-1-R; AAAAATGTGGTTGCCTCTGA, ARD-2-R; GGACTG-GAGTGCTCTATGAATTG, and ARD-3-R; TGTCAGCAGCC-TACCACTAGC) primers were designed using the Primer3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Operon Biotecnologies Inc., Huntsville, AL). Anticipated PCR products using combinations of these primers ranged between 370 and 390 bp, depending on the primer combinations. The criteria for the primers were a product size of between 200 and 400 bases, a primer size of 18–27 bases with a minimum GC% of 20% and a maximum of 80%, and a Tm of between 57 and 63 °C. Primers were tested in all possible combinations against selected samples (WM, VM, RM, RS 2, VS 3, WS10, VSm 1, VSm 2, and VSm 3), and then samples were tested by primer combinations of ARD-1-F and ARD-4-R and of ARD-1-F and ARD-5-R at annealing temperature of 55 °C.

3. Results

3.1. Characterization based on the morphological characteristics

Young leaves of 45 RS and 21 WS at seedling stage were not morphologically distinguishable from each other and from their mother plants, RM and WM, respectively (Fig. 2). Leaves of seven VSm seedlings, however, were not undulated with wavy margins that wind up and down and appeared to be very similar to leaves of RM and WM. However, color composition of the leaf blade of a selected VSm seedling 3 (VSm 3, Fig. 1A) was similar to WM, the red color of petiole is similar to WM, and the red color of the tip is similar to RM and VM when young shoot tips of WM, RM, VM, and a selected VSm seedling 3 (VSm 3) were scanned and color components for red, green, and blue from leaf, petiole, tip, and stem was recorded from three points for red/green/blue readings using Jasc Paint Shop Pro 7 (Jasc Software, Eden Prairie, MN) (data not presented).

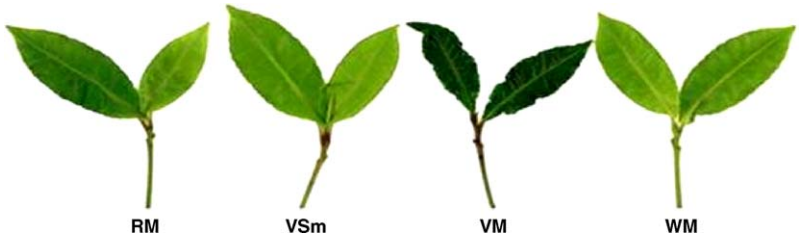

Fig. 2. Shoot tips with two terminal leaves of *A. crenata* with regular foliage and red berries (RM), seedling with non-variegated foliage with reddish shoot tip (VSm 3), with variegated foliage and red berries (VM), with variegated foliage and white berries (WM).

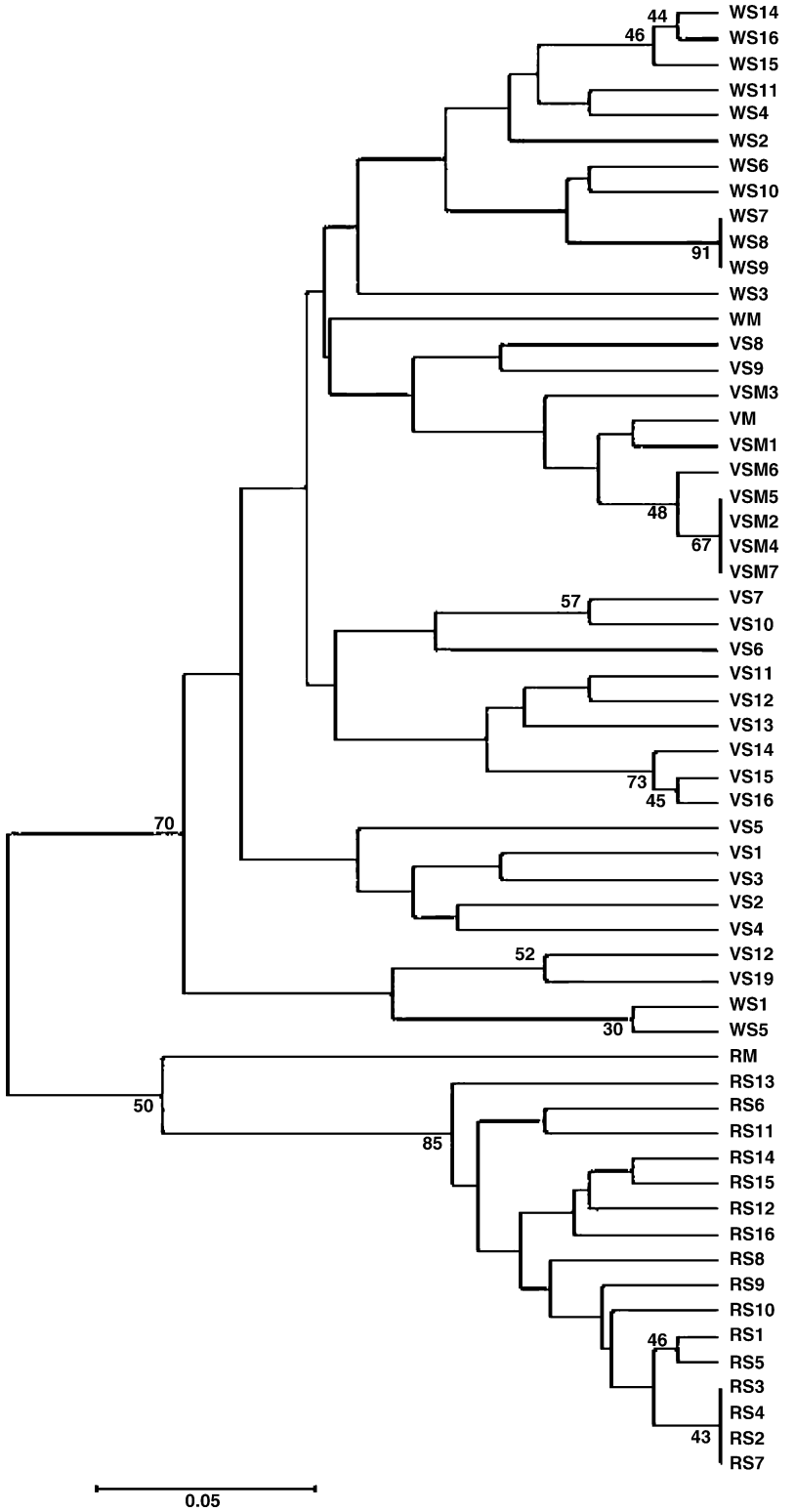

Fig. 3. A dendrogram of *Ardisia* mother plants and seven seedlings. RM, WM, and VM are the mother plants and RS, WS, and VS indicate seedlings with the same leaf morphology as in RM, WM, and VM. VSm indicates seedlings with non-variegated foliage. Bootstrap values lower than 40% are not shown.

3.2. Molecular marker assisted characterization

Mother plant with red berries (RM) and its seedlings (RS 1–16) clustered together in one major cluster which was distinct from WM, all WS, VM, and VSm (Fig. 3). This separation is supported by the high bootstrap values for the RM and RS (RM/RS) branches. Although WM and the majority of WS, and also VM and VSm clustered in another major group, some WM seedlings (WS 1, 5, 12, and 13) exhibited considerable variations and were clustered close to RM and RS, and appear

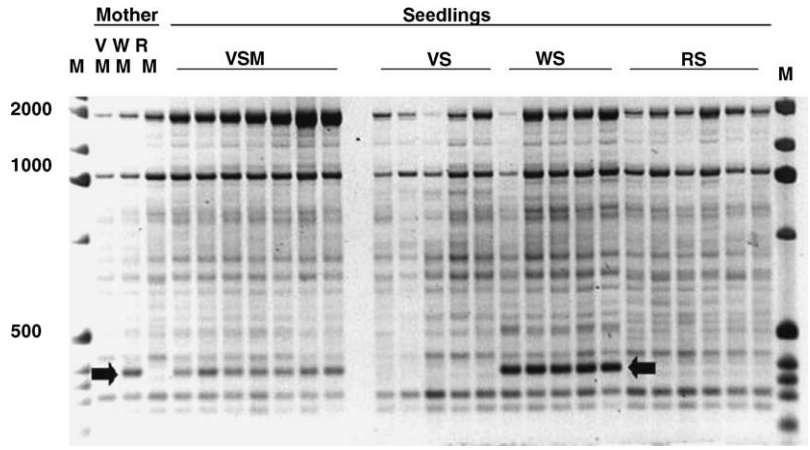

Fig. 4. A RAPD gel photo obtained using OPB-04 primer of selected RS, WS, RS, and seven VSm seedlings with mother plants (RM, WM, VM). M: molecular size of DNA.

slightly basal to the WM/WS and VM/VS group. It is interesting to note that VM, and all seven VSm were clustered closely together. However, other VS seedlings were not clustered together with VM and VSm. Since all of the WM and VM and progeny can be reduced to one large polytomy because only six of the branches have support greater than 50, it appears that RAPD with the primers tested was unable to resolve WM, VM, and their progenies.

The WM-specific band produced with OPB-04 primer was cloned (Fig. 4) and sequenced for WM, two WM seedlings, and VSm 4. Based on the 374-base sequence (Table 1), the single nucleotide substitutions (SNS) revealed that WM and VSm 3 yielded the same nucleotide sequence (Table 1). However, individual clones from VSm 4, 4–2, and 4–5, showed SNS at the positions 73 (C) and 243 (A), respectively. clones clones from WS 3 and five clones of WS 5 showed the same sequence as in the mother plant, WM. Seven clones of WS 5 showed a substitution or deletion at various positions.

3.3. Confirmation of hybrid origin of VSm using WM specific SCAR markers

Hybrid origin of VSm seedlings between VM as a maternal source and WM as a paternal source is confirmed by PCR utilizing SCAR markers. Forward primer, ARD-1-F paired with each of two reverse primers, ARD-2-R and ARD-3-R,

Table 1
Single nucleotide substitutions at various positions

SNS position[a]	Mother plant or seedlings – colony												
	WM	VSm 4[b]	VSm 4-2	VSm 4-5	WS 3[c]	WS 5[d]	WS 5-2	WS 5-3	WS 5-4	WS 5-8	WS 5-9	WS 5-22	WS 5-24
73	T	T	***C***[e]	T	T	T	T	T	T	T	T	T	T
97	T	T	T	T	T	T	***C***	T	T	T	T	T	T
119	G	G	G	G	G	G	G	***A***	G	G	G	G	G
165	T	T	T	T	T	T	T	T	T	T	T	***A***	T
243	T	T	T	***A***	T	T	T	T	T	T	T	T	T
248	T	T	T	T	Y	T	T	T	–[f]	T	T	T	–
271	G	G	G	G	G	G	G	G	G	G	***A***	G	G
279	A	A	A	A	A	A	A	A	A	***G***	A	A	A
284	T	T	T	T	T	T	T	T	T	T	**C**	T	T
302	T	T	T	T	T	**C**	T	T	T	T	T	T	T

Three colonies (minimum) that do not have colony numbers to 12 colonies (maximum) from each plant were sequenced; number of clones ([b,c,d]) that yielded the same single nucleotide sequences.

[a] A 374-base consensus nucleotide sequence data for mother plants (WM) and seedlings (WS) of *A. crenata*. Nucleotides in bold and italic letters indicate primer sites.
GGACTGGAGT AGAGGATAGA GTTTTGAGTT TATTTTTTTC TTTGTTATTT TTTTTAATTT ATTTTTTTCT TTGGTTGGAG
TTAGAGTTTT TCAGCAGTTA GTTTTGTTAG ACCTCCAATT CTGACCACTT TCATTTTTTT TGTAAACTCT TGTATTCATA
CCAGTTTTCA GTTCATAATC AGTAATAGAA GTATTTCATA AGTATAATTT CTCTTTATAG TTCCAATTGT TCTTCCAATT
TTTTTTTATA TAAGATCAGA GGCAACCACA TTTTTTTCCT AAAGCTTTAG GTTTTTTTTT TTGGTTCTGT TTTTTTTTTT
CTTTTATTTT TAGCTAGTGG TAGGCTGCTG ACAATTCATA GAGC***ACTCCA*** ***GTCC***.

[b] VSm-six clones.
[c] WS-11 clones.
[d] WS-five clones.
[e] Substituted nucleotide base.
[f] Deletion.

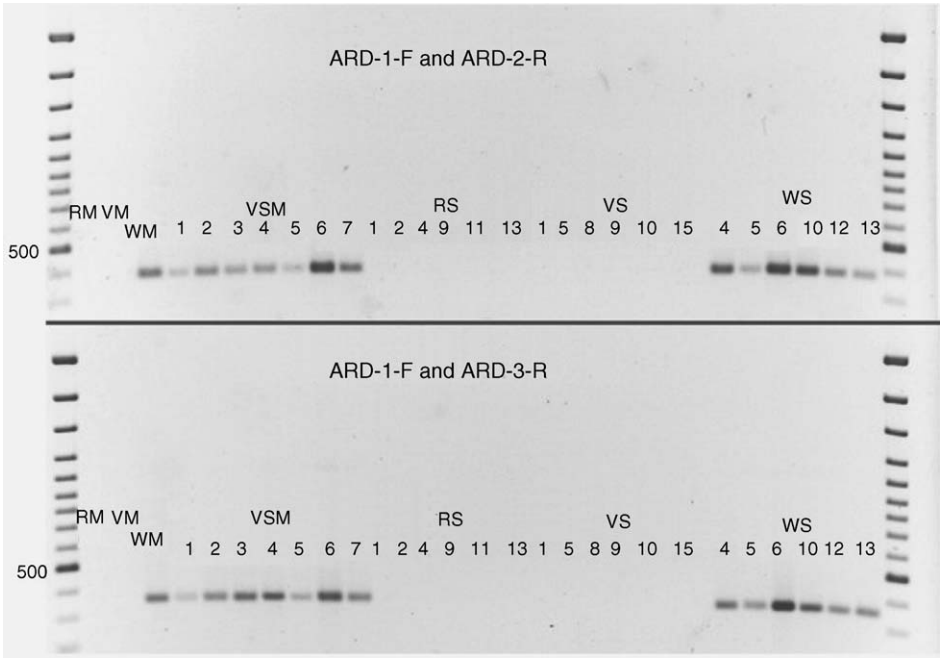

Fig. 5. Primer combination sets of ARD-1-F; GGACTGGAGTAGAGGATAGAGTTTTG and ARD-2-R;GGACTGGAGTGCTCTATGAATTG, and of ARD-1-F and ARD-3-R; TGTCAGCAGCCTACCACTAGC selected for testing samples of RM, WM, WM, VSm 1–7, RS 1, 2, 4, 9, 11, and 13, and VS 1, 5, 8, 9, 10, and 11, and WS 4, 5, 6, 10, 12, and 13 at annealing temperature of 55 °C.

amplified DNA in VSm, WM, and WS plants; neither VM, RM nor any RS and VS seedlings yielded PCR products with these primers (Fig. 5). Anticipated product size of ARD-1-F and ARD-2-R and of ARD-1-F ARD-3-R was 374 and 353 bp, respectively.

4. Discussion

4.1. Characterization based on the morphological characteristics and RAPD markers

Based on the morphological characteristics, the possibility of a hybrid origin of VSm with RM was suggested, as parental source for its reddish petioles and shoot tip, but the reddish color could also be inherited from VM even if it hybridized with WM. The possibility of segregation, chimera, or mutation from the self-pollinated plants for the origin of VSm must also be considered because other VS seedlings were not clustered closely with VM. As of March, 2006, four VSm plants produced berries and color of berries of VSm 3 (two berries) and VSm 5 (three berries) are red and of VSm 6 (3 berries) and VSm 7 (3 berries) are white. Berry color of VSm 6 showed very faint orange-red color during seed maturation. The lack of variegation, the reddish color in the crown, and the variation in berry color all suggest that the rare VSm phenotype is considered to be of hybrid origin.

Based on the dendrogram, RM is probably not the pollen source, and cross pollination between VM and WM might have occurred, or VM might be segregated from WM. Further, it appears that VM and WM, and thus VS and WS, share a great deal of genetic background, making it nearly impossible to conclude that VSm are hybrids based on RAPD alone. However, the separation between RM/RS and VSm suggests that RM is not the paternal source for VSm. The presence of a RAPD band of approximately 390 bp long band produced in all seven VSm seedlings, all the WS seedlings and in the WM, but not in VM indicated that RM could be effectively eliminated by the RAPD data as a parent. Therefore, it is concluded that these seven VSm seedlings resulted from a cross-pollination between VM and WM as paternal parent, which is further supported by the low outcrossing rate observed in a small population of *A. crenata* var. *bicolor* (Chen et al., 2001). The WM-specific marker band could be used to effectively screen for leaf morphology, or hybrid origin, among VM progeny. However, it is not effective for predicting of red or white berry color. We hope that by testing more primers or utilizing other techniques, it could become possible to identify markers that are linked to fruit characteristics as reported in the interspecific Asian pears (Kim et al., 2004).

4.2. Confirmation of hybrid origin of VSm using WM specific SCAR markers

Sequence data analysis of the WM-, WS-, and VSm-specific band produced with OPB-04 primer may suggest polymerase error unless it is a plastid locus present in multiple copies or presence of mutations in sequences. Morphological characteristics of foliage and color at the shoot tips of WS 5 were not different from WM and the rest of seedlings derived from WM. Therefore, SNS data alone may not be effective or useful to explain hybrid origin of VSm or comparisons between plants. Therefore, specific primers were designed for diagnostic assay to examine whether products resulted from VSm, WM, and WS.

SCAR markers developed in this study can be used using the described PCR conditions to test other potential hybrids

involving WM as a parent. As shown in the dendrogram constructed from RAPD markers, the origin of the hybrid except to suggest that RM is probably not the pollen source. In *Brassica*, two linked AFLP markers converted to SCAR were utilized to develop yellow-seeded lines by means of marker-assisted selection (Sabharwal et al., 2004). Characterization of VSm seedlings for red berry or white berry coloration, however, is not possible using these specific primer sets since VSm seedlings that produced red or white berries produced the RAPD marker band. These primers appear to identify a WM-specific DNA fragment that can be used to verify hybrid origin of plants crossed with WM. For the VM × WM cross, the male contribution to the seedling also appears to confer non-variegated status to the leaves, at least in seven of the seven hybrids detected. However, there is no evidence that this PCR assay is specific to variegated versus non-variegated leaves in WM hybrids since it was not tested on a segregating population. Blast analysis (Altschul et al., 1997) of the sequences did not result any significant matches for nucleotide and no match for protein in GenBank.

5. Conclusion

The mechanism of pollination, and whether *A. crenata* is self-pollinated or cross-pollinated, is not well understood at present. Based on the presence of a specific RAPD band for VSm, WM, and WS, and utilization of specific primers, it is concluded that VSm seedlings are result from cross pollination between VM and WM. Therefore, cross pollination is a source of creation of new hybrids in *A. crenata*. Hybrid origin of VSm seedlings between VM as a maternal source and WM as a paternal source was verified by polymerase chain reaction (PCR) utilizing SCAR markers (forward primer, ARD-1-F and two reverse primers, ARD-2-R and ARD-3-R).

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